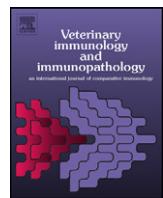




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Technical report

Sequence analysis, characterization and mRNA distribution of channel catfish (*Ictalurus punctatus* Rafinesque, 1818) chemokine (C-X-C motif) receptor 4 (CXCR4) cDNA

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ABSTRACT

Chemokine receptor CXCR4, a member of the G protein-coupled receptor superfamily, binds selectively CXCL12. This protein plays many important roles in immunological as well as pathophysiological functions. In this study, we identified and characterized the channel catfish CXCR4 transcript. The full-length nucleic acid sequence of channel catfish CXCR4 cDNA comprised of 1994 nucleotides, including an open reading frame, which appears to encode a putative peptide of 357 amino acid residues with a calculated molecular mass of 40.1 kDa. By comparison with the human counterpart, the channel catfish CXCR4 peptide can be divided into domains, including seven transmembrane domains, four cytoplasmic domains, and four extracellular domains. The CXCR4 transcript was detected in spleen, anterior kidney, liver, intestine, skin and gill of all catfish examined in this study. Because four CXCL of channel catfish have been identified, the result provides valuable information for further exploring the channel catfish chemokine signalling pathways and their roles in immune responses to infection.

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Chemokines (also called chemotactic cytokines) are a superfamily of small structurally related, proinflammatory proteins (Baggiolini, 1998; Hedrick and Zlotnik, 1996; Mantovani, 1999; Yoshie et al., 2001). Upon their binding to the chemokine receptors on the cell surface, the complexes initiate a series of intracellular signalling pathways, resulting in various physiological and pathological processes, such as immune responses and surveillance, tissue repair and regeneration, or cancer metastases (Adler et al., 2006; Allen et al., 2007; Watt and Forde, 2008). In human 48 chemokines and 20 chemokine receptors have been characterized and classified into four families (C, CC, CXC and CX3C) based on the arrangement of their first two conserved cysteine residues in chemokines (Zlotnik and Yoshie, 2000). Recently, corresponding chemokines have

also been identified in channel catfish (Baoprasertkul et al., 2004, 2005; Bao et al., 2006; Peatman and Liu, 2007): 28 CC and four CXC chemokines. However, only a few fish chemokine receptors has been identified and characterized—CXCR4 and CCR7 of rainbow trout (Daniels et al., 1999), CXCR4 of common carp (Fujiki et al., 1999), CXCR4 of sterlets (Alabyev et al., 2000), CXCR4 of lamprey (Kuroda et al., 2003) and CCR1–9 of zebrafish (Liu et al., 2009).

CXCR4, the seven-spanning hydrophobic transmembrane protein, is a member of the G protein-coupled receptor superfamily (Murphy et al., 2000), and its ligand is a stromal cell-derived factor (also known as CXCL12 chemokine) (Murphy et al., 2000; Fredriksson et al., 2003). Because CXCR4 on the human T cell surface mediates HIV entry (Doranz et al., 1996; Feng et al., 1996), CXCR4 has been extensively studied in relation to its structure, signalling pathways and targets for inhibitor development for controlling HIV infection (for reviews, see Busillo and Benovic, 2007; Allen et al., 2007; Liang, 2008; Patrucci and

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Table 1

Synthetic oligonucleotide primers used in this study.

Primer	Sequence	Direction
GeneRacer 5'-primer (Invitrogen)	5'-CGACTGGAGCACGAGGACACTGA-3'	Forward
GeneRacer 3'-primer (Invitrogen)	5'-GCTGTCAACGATACGCTACGTAACG-3'	Reverse
β-Actin-F	5'-GACTTCGAGCAGGAGATGGG-3'	Forward
β-Actin-R	5'-AACCTCTATTGCCAATGGT-3'	Reverse
CXCR4-170F	5'-ATCTCCGGTCAAGCCAGGACTTCAA-3'	Forward
CXCR4-295F	5'-CTCATCTGCCTTCATCAGCCTGGA-3'	Forward
CXCR4-639F	5'-GAGGAAGGCGCTGAAGACCACCATCA-3'	Forward
CXCR4-366R	5'-CCTCGTCGCCTGGCTTTGTAGCAC-3'	Reverse

Baldari, 2008; Thelen and Thelen, 2008; Watt and Forde, 2008; Miller et al., 2008). Recently, several studies in zebrafish demonstrated that fish CXCR4 involves in early embryonic development, in primordial germ cell migration and in skeletal myogenesis (Chong et al., 2007; Valentini et al., 2007; Mizoguchi et al., 2008; Sasado et al., 2008).

In this communication, we describe the isolation, characterization and analysis of tissue expression of channel catfish CXCR4 transcript.

Channel catfish (NWAC 103 strain) were used in this study according to the Guidelines for the Use of Fish in Research (Nickum et al., 2004). The protocol for experimental use of catfish was approved by the Institutional Animal Care and Use Committee, Aquatic Animal Health Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Auburn, AL.

Total RNA from channel catfish tissues was purified by using a Tri reagent (Molecular Research Center, Inc., Cincinnati, OH) per the manufacturer's instructions. The quality and quantity of total RNA were determined with an Agilent Bioanalyzer using RNA 1200 chips (Agilent Technologies, Santa Clara, CA). Both 5'- and 3'-RACE libraries were generated by using a GeneRacer kit (Invitrogen, Carlsbad, CA) according to the protocol provided in the kit. Oligonucleotide primers for PCR amplification are listed in Table 1. The amplified PCR

fragments were cloned into a pSC vector (Agilent Technologies) per the manufacturer's instructions. At least six colonies per PCR product were randomly selected for sequencing.

DNA sequencing on both strands was carried out at the USDA ARS Genomics and Bioinformatics Research Unit (Stoneville, MS) as described previously (Yeh and Klesius, 2007a,b, 2008a,b,c). The amino acid sequence was deduced by using the Transeq program (Rice et al., 2000), and aligned with other CXCR4 amino acid sequences deposited in GenBank by using ClustalW2 software (Larkin et al., 2007). ExPASy server (Gasteiger et al., 2005) was used to calculate the CXCR4 peptide molecular mass and pI. Transmembrane topology and signal peptide of the CXCR4 peptide were predicted via Phobius Web server (Käll et al., 2007). Phylogenetic relationships of CXCR4 amino acid sequences from various species were analyzed with the neighbor-joining method by using the MEGA 4.0 software (Tamura et al., 2007) based on the alignment results from ClustalW2. The tree was built on 1000 bootstrap replicates with the value >50%.

RT-PCR assays for detection of CXCR4 in channel catfish ($n=4$) tissues were carried out by a two-step procedure described previously (Yeh and Klesius, 2007a,b, 2008a,b,c). The transcripts were amplified for 40 cycles at 98 °C for 10 s, 62 °C for 1 min and 72 °C for 1.5 min. The β-actin

Table 2Identity of CXCR4 amino acid sequences among species in percentage^a.

	Channel catfish	Common carp	Zebrafish	Spotted green pufferfish	Rainbow trout	Turbot	Atlantic salmon	Sterlet	African clawed frog	Chicken	Rat	Pig	Cattle	Horse	Human
Common carp	77														
Zebrafish	74	88													
Spotted green pufferfish	70	70	69												
Rainbow trout	69	70	68	72											
Turbot	70	70	69	79	75										
Atlantic salmon	67	73	72	74	87	74									
Sterlet	64	68	64	65	65	67	67								
African clawed frog	60	64	60	62	61	62	63	63							
Chicken	60	60	59	64	64	62	62	67	76						
Rat	62	61	59	63	64	63	62	66	74	79					
Pig	61	61	60	65	60	63	61	66	72	79	89				
Cattle	62	61	61	65	61	62	61	66	71	79	87	95			
Horse	61	60	59	65	60	62	60	66	72	78	89	97	94		
Human	61	60	59	65	61	62	61	66	74	81	90	94	92	93	
Dog	61	61	59	65	60	62	62	66	72	79	88	95	93	94	95
Cat	61	61	60	65	60	63	61	66	73	79	89	98	94	96	94
															97

^a Identity between two species was calculated by the ClustalW2 software (via www.ebi.ac.uk).

transcript was used as an internal control. Images were documented by a Kodak Gel Logic 440 Imaging System (Eastman Kodak Co., Rochester, NY), and processed using the ImageJ software (Abramoff et al., 2004).

Our preliminary study by subtractive suppression hybridization identified one expressed sequence tag (EST) of channel catfish CXCR4. Based on this EST, we designed gene-specific primers in conjunction the Gen-eRacer primers (Table 1) to amplify both 5'- and 3'-end

cDNA of channel catfish CXCR4 (Frohman et al., 1988). The full-length of channel catfish CXCR4 cDNA had 1994 nucleotides, including 78 nucleotides at 5'-untranslated region (UTR), 1074 nucleotides of an open reading frame (ORF) and 842 nucleotides at 3'-UTR (GenBank accession no. GQ169128). A Kozak sequence (atcatgg) (Kozak, 1980) was found in the 5'-UTR. In the 3'-UTR, three canonical features of mRNA were identified: (1) four mRNA instability motifs at positions 1351–1355, 1509–1513,

Fig. 1. Multiple sequence alignment of the deduced channel catfish CXCR4 with those from other species deposited in GenBank. Gaps (–) were introduced to maximize the sequence homology. Identical amino acids among all species were indicated by asterisks (*) below the sequences. The structural domains were also indicated above the sequences. Characteristic conserved motif and amino acids were highlighted. GenBank accession numbers for each species are following: African clawed frog, NP_001080681; Atlantic salmon, ACN10355; cat, NP_001009826; cattle, NP_776726; channel catfish, GQ169128; chicken, NP_989948; common carp, BAA32797; dog, NP_001041491; horse, XP_001490215; human, AAP36716; pig, NP_998938; rainbow trout, NP_001117814; rat, O08565 spotted green pufferfish, CAG01848; starlet, CAB60252 turbot, ABP48751; and zebrafish, AAH95021.

	membrane 4	Extracellular Domain	Trans-
Pig	LLLTIPDFIFANVRE	-GDGRYICDRFYPNDL--WLVVVFQFQHIMV	207
Horse	LLLTIPDFIFANVRE	-GDGRYICDRFYPSDL--WLVVVFQFQHIMV	207
Cattle	LLLTIPDLIFADIKE	-VDERYLICDRFYPSDL--WLVVVFQFQHIVV	207
Cat	LLLTIPDFIFANVRE	-ADGRYICDRFYPSDS--WLVVVFQFQHIMV	207
Dog	LLLTIPDFIFANVRE	-ADDRLYICDRFYPNDS--WLVVVFQFQHIVV	207
Human	LLLTIPDFIFANVRE	-ADDRLYICDRFYPNDL--WLVVVFQFQHIVV	206
Rat	LLLTIPDIIIFADVSQ	-GDGRYICDRLYPSDL--WMVVVFQFQHIMV	203
Chicken	VLLTVPDIIIFASTSE	-VEGRYLICDRMYPHDN--WLISFRFQHILV	212
African clawed frog	LLLTVPDLVFAVSVN	-ENGQFCVCDRIVYIDNRETWTGFRFLHITV	213
Sterlet	TLLTVPDLVFAQVHD	-EGTRMMICDRVYPSESCGNIWMTIFRFQHIVF	211
Common carp	SLLTVPDLVFAKVHD	-TGMLTICELTYPLQGNTVWKAVFRFHQHIVF	207
Zebrafish	TFFTIPDLVFAKIHN	-SSMGTCICELTYPQEANVIWKAVFRFHQHIII	207
Channel catfish	MLMTIPDLVFAKVQS	-TGTKNICDRIYPHEGNMVMWKAVFHFQHILV	211
Spotted green pufferfish	AVLTVPDLVFARVQSSGSSNIDLFEENMETAQSRILICQRYIPEETSLIWIWAVFRFHQHILV	229	
Turbot	ALLTVPMVFRVAKH--	-KYHTDPSMDTAESRTICQRIYPOETSFOWATAASRFQHILV	224
Rainbow trout	VILTVPDIVFATALD-----	-GGSRTICQRIYPOQKTSFYWMAGFRFHQHILV	211
Atlantic salmon	AVLTVPDIVFATALD-----	-SGSRTICQRIYPOQKTSFYWMAAFRFQHILV	216
	* * *	* * *	* * *
	Membrane 5	→ Cytoplasmic Domain	Transmembrane 6 →
Pig	GLILPGIVILSCYCIIISKLSH-SKGYQ-KRKALKTTVILILAFFACWLPLYIGISIDSF	265	
Horse	GLILPGIVILSCYCIIISKLSH-SKGYQ-KRKALKTTVILILAFFACWLPLYIGISIDSF	265	
Cattle	GLILPGIVILSCYCIIISKLSH-SKGYQ-KRKALKTTVILILAFFACWLPLYIGISIDSF	265	
Cat	GLILPGIVILSCYCIIISKLSH-SKGYQ-KRKALKTTVILILAFFACWLPLYIGISIDSF	265	
Dog	GLILPGIVILSCYCIIISKLSH-SKGYQ-KRKALKTTVILILAFFACWLPLYIGISIDSF	265	
Human	GLILPGIVILSCYCIIISKLSH-SKGHQ-KRKALKTTVILILAFFACWLPLYIGISIDSF	264	
Rat	GLILPGIVILSCYCIIISKLSH-SKGHQ-KRKALKTTVILILAFFACWLPLYIGISIDSF	261	
Chicken	GLVLPGLIILTCYCIIISKLSH-SKGHQ-KRKALKTTVILILAFFACWLPLYVGISIDTF	270	
African clawed frog	GLVLPGLIILTCYCIIISKLSH-SKGHQ-KRKALKTTVILILAFFACWLPLYVGISIDTF	271	
Sterlet	GLVLPGLVILTTCYCIIITKLSQLGSKGLQ-KRRAKALTTIILILAFFCWLPCYIALVDTL	270	
Common carp	GFLLPGLIILTCYCIIISKLKSNSKGQLAKRALKTTVILILCFICWLPCYAGILVDTL	267	
Zebrafish	GFLLPGLIILTCYCIIISKLKSNSKGQLTAKRALKTTVILILCFICWLPCYAGILVDTL	267	
Channel catfish	GFVPLGLVILICYCIIISKLSKGSKGQALKRALKTTIILVLCFFVCWLPCAGILVDTL	271	
Spotted green pufferfish	GFVPLGLVILICYCIIISKLSQLGAKGQALKKKALKTTVILILCFSCWLPCVGIFLDTL	289	
Turbot	GFVPLGLVILICYCIIIAKLSQGAKAQLAKKKALKTTVILIVCFGCWLPCYGLIFLDTL	284	
Rainbow trout	GFVPLGLVILTCYCIIIAKLSQGAKGVQLKRALKTTVILILCFSCWLPCVGIFLDTL	271	
Atlantic salmon	GFVPLGLVILTCYCIIIAKLSQGAKGVQLKRALKTTVILILCFSCWLPCVGIFVDTL	276	
	* * * * *	* * * * *	* * * * *
	Extracellular Domain	→ Transmembrane 7 →	Cytoplasmic
Pig	ILLEIIQGCEFESTVHKWISITEALAFFHCCLNPILYAFLGAKFKTSQAQHALTSVSRGS	325	
Horse	ILLEIIQRGCEFESTVHKWISITEALAFFHCCLNPILYAFLGAKFKTSQAQHALTSVSRGS	325	
Cattle	ILLEIIQGCEFESTVHKWISITEALAFFHCCLNPILYAFLGAKFKTSQAQHALTSVSRGS	325	
Cat	ILLEIIQKGCEFESTVHKWISITEALAFFHCCLNPILYAFLGAKFKTSQAQHALTSVSRGS	325	
Dog	ILLEIIQKGCEFEKTVHKWISITEALAFFHCCLNPILYAFLGAKFKTSQAQHALTSVSRGS	325	
Human	ILLEIIQKGCEFENTVHKWISITEALAFFHCCLNPILYAFLGAKFKTSQAQHALTSVSRGS	324	
Rat	ILLEVIKGQGCEFESVHKWISITEALAFFHCCLNPILYAFLGAKFKTSQAQHALNSMRGS	321	
Chicken	ILLGVIRHRCSDLTIVHKWISITEALAFFHCCLNPILYAFLGAKFKTSQAQNALTTSVSRGS	330	
African clawed frog	MMLGLVRACKDWTENTLHKAA SITEALAFFHCCLNPILYAFLGAKFKTSQAQNAFTSVSRGS	331	
Sterlet	VLLNVIQYNCTLQHMETWIVTEGLAYFHCCLNPSILYAFLGVFKFKSAKSALTIVNSRGS	330	
Common carp	VMLNVISHSCFLEQGLEKWIFFEALAYFHCCLNPILYAFLGVFKFSKSARNALSISSRSS	327	
Zebrafish	TMLNVISHSCFLEQGLEKWIFFEALAYFHCCLNPILYAFLGVRFSKSARNALSISSRSS	327	
Channel catfish	VTLNLSVGCKLERGLQKWLITEALAYFHCCLNPILYAFLGVRFSKSARSALS VNSRSS	331	
Spotted green pufferfish	MLLNVNVSSPCGLQHAVEKWISITEALAYFHCCLNPILYAFLGVFKFKSAKNALTS--RSS	347	
Turbot	MMLNVRSSCELQQAIVEKWISITEALAYFHCCLNPILYAFLGVFKFKTARTALTIVSSRSS	344	
Rainbow trout	MLLNVISHSCALEQLSQLTWLLITEALAYFHCCLNPILYAFLGVFKFKSARDALAVNSSSS	331	
Atlantic salmon	MLLNVISHNCALEQLSQLTWLLITEALAYFHCCLNPILYAFLGVFKFKSARNALTIVSSRSS	336	
	* *	* * * * *****	* * * * *
	Domain	→	
Pig	SLKILSKGKRGHHSSVSTESESSSSFHSS	353	
Horse	SLKILSKGKRGHHSSVSTESESSSSFHSS	353	
Cattle	SLKILSKGKRGHHSSVSTESESSSSFHSS	353	
Cat	SLKILSKGKRGHHSSVSTESESSSSFHSS	353	
Dog	SLKILSKGKRGHHSSVSTESESSSSFHSS	353	
Human	SLKILSKGKRGHHSSVSTESESSSSFHSS	353	
Rat	SLKILSKGKRGHHSSVSTESESSSSFHSS	349	
Chicken	SLKILSKGKRGHHSSVSTESESSSSFHSS	358	
African clawed frog	SLKILSKRAGLSSSVSTESESSSSFHSS	358	
Sterlet	SLKILSKRNKRGGLSSSVSTESESSSSFQSS	358	
Common carp	HMM-LTKKRGPISSSVSTESESSSSVLISS	353	
Zebrafish	HMM-LTKKRGPISSSVSTESESSSSVLISS	353	
Channel catfish	QKF-LTKKRGHVSSVSTESESSSSVLISS	357	
Spotted green pufferfish	QKATLMTKKRGPISSSVSTESESSSSVLISS	375	
Turbot	QKVNLMTKKRGAISSSVSTESESSSSVLISS	372	
Rainbow trout	HKV-LTKKRGAISSSVSTESESSSSVLCIS	357	
Atlantic salmon	HKV-LTKKRGPISSSVSTESESSSSVLYS	362	

Fig. 1. (Continued).

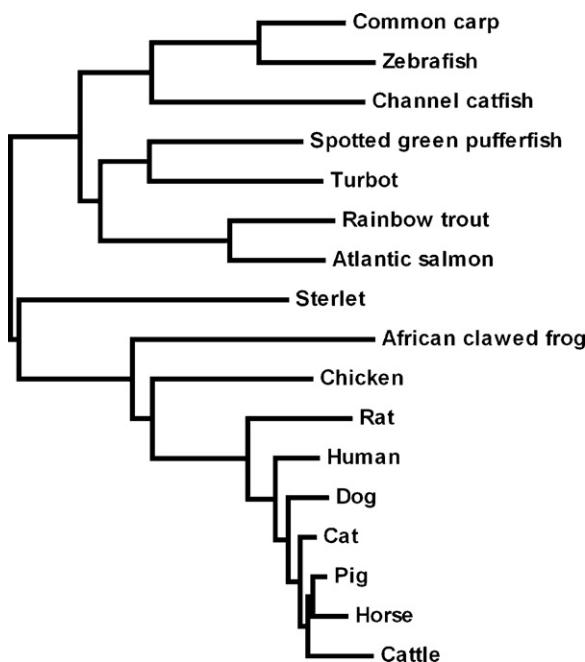


Fig. 2. Phylogenetic relationships of the deduced channel catfish CXCR4 with other species. The sequences for the phylogenetic analysis, which was obtained by using the alignment results from ClustalW2, are the same as those in Fig. 1.

1690–1694 and 1868–1872, (2) a polyadenylation signal sequence at position 1948–1953, and (3) a 28-nucleotide polyadenylation tail (41 nucleotides downstream of the polyadenylation signal sequence). These features indicate this channel catfish CXCR4 cDNA is in full-length. The ORF appears to encode a 357 amino acid peptide with a calculated molecular mass of 40.1 kDa and pI of 9.06 at pH 7.0. Like human CXCR4 (Berson et al., 1996), channel catfish CXCR4 had two potential N-glycosylation sites at Asn¹³ and Asn⁷³.

When the predicted channel catfish CXCR4 amino acid sequence was compared with those of other species deposited in the GenBank database, we noticed that the catfish CXCR4 was 64–77% identical among fish species,

while was 61–62% identical to the mammals (Table 2). The high identity of the amino acid sequences is observed in the transmembrane domains (Fig. 1). In addition, the catfish CXCR4 can be divided into seven hydrophobic transmembrane domains (TM1–TM7), four extracellular and four intracellular domains (Fig. 1). Several important conserved features can also be found in catfish CXCR4: (1) The chemokine receptor-specific motif (DRYLAVRA) in cytoplasmic domain after TM3 except two underlined amino acids is conserved among mammals and teleost fish. (2) Seven cysteine residues, which may form disulfide bonds to stabilize the binding pocket of the receptor (Kuroda et al., 2003), at positions 30, 223, 225, 268, 281, 302 and 303 with a CXC motif (²²³C-²²⁴Y-²²⁵C) in TM5 are conserved in channel catfish CXCR4. In our previous reports, we have found that conservation of disulfide bonds exist in many channel catfish deduced peptides, such as CD59, CD81, CD156a, UCHL5 and cathepsins (Yeh and Klesius, 2007b, 2008a, 2009a,b,c,d). (3) Except in TM3, the proline residue is conserved in each TM of 17 sequences examined. (4) The lysine residue, which is important for ligand degradation (Marchese and Benovic, 2001), at positions 333, 337 and 338 is conserved in channel catfish. (5) Like mammalian CXCR4, catfish CXCR4 had 11 serine residues in the carboxyl terminus (cytoplasmic domain), suggesting that these serine residues may involve in CXCR4 phosphorylation and internalization after ligand binding (Haribabu et al., 1997; Signoret et al., 1997, 1998; Orsini et al., 1999).

Phylogenetic analysis based on the amino acid sequence alignment demonstrated two distinguishable evolutionary clusters (Fig. 2). The channel catfish CXCR4 falls in the clustering with other fish species and away from the mammalian counterparts, consistent with classical taxonomy and phylogenetic transition. These results are in agreement with our previous studies in other channel catfish genes, such as CD59, CD81, cathepsins, cyclophilin A and B, matrix metalloproteinase-9, and peroxiredoxin-6 (Yeh and Klesius, 2007a,b, 2008a,b,c, 2009b,c).

The CXCR4 expression profile in channel catfish tissues was examined by the two-step multiplex RT-PCR assays. The amplified CXCR4 and β-actin PCR products had 359 and 203 nucleotides, respectively. As seen in Fig. 3, the

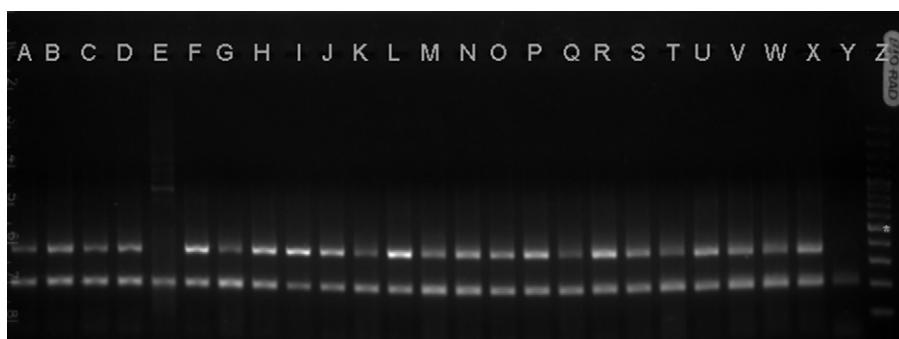


Fig. 3. Tissue distribution of channel catfish CXCR4 transcript ($n = 4$). Total RNA from various tissues was used for RT-PCR assays. The amplified PCR products were analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining. The sizes of amplified CXCR4 and β-actin were 359 and 203 nucleotides, respectively. Spleen (lanes A, G, M and S), anterior kidney (lanes B, H, N and T), liver (lanes C, I, O and U), intestine (lanes D, J, P and V), skin (lanes E, K, Q and W), and gill (lanes F, L, R and X). Lane Y, no template control, and lane Z, 100-bp molecular weight ladders (Fermentas Life Sciences, Glen Burnie, MD). * Indicates 500 bp.

channel catfish CXCR4 gene transcript was detected in spleen, anterior kidney, liver, intestine and gill of fish examined, suggesting that the CXCR4 transcript is constitutively expressed and may be important in immune surveillance and other physiological functions. These patterns of tissue distribution in channel catfish are in agreement with the reports from other species (Rimland et al., 1991; Loetscher et al., 1994; Daniels et al., 1999; Liang et al., 2001; Kuroda et al., 2003). The CXCR4 expression in skin was not detected in one fish and low in other fish. The reason is not known, but it may be due to cell types and cell number in skin. Reactions without cDNA template did not show amplification (Fig. 3, lane Y).

In conclusion, based on sequence alignment and phylogenetic analysis, the full-length of the channel catfish CXCR4 transcript was identified. The transcript was constitutively expressed in spleen, anterior kidney, liver, intestine, skin and gill of fish examined. Because four CXCL (ligands) of channel catfish have been identified (Baoprasertkul et al., 2004, 2005), this result provides valuable information for further exploring the channel catfish chemokine signalling pathways and their roles in immune responses to infection.

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